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Lenticules for the control of quantitative methods in food microbiology

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A.A. CODD, I.R. RICHARDSON AND N. ANDREWS. 1998. Control dried organisms as lenticules are a dependable and convenient alternative to wet cultures for quality assurance and process controls in routine food microbiology. Lenticules are designed to give a fixed, reproducible inoculum over an extended period of time without loss of cultural characteristics or viability. During a period of 23 months, 596 paired counts were performed by both Miles and Misra and spiral plating techniques on lenticule controls. Correlation between the two methods and within batches was excellent. Only 14 counts (2·5%) fell outside the standard operating limit of 0·5 $\log_{10}$. All were within 1·0 $\log_{10}$. On two separate occasions, replicate runs were performed on five reconstituted lenticules from a batch. The counts obtained showed variation within and between lenticules only slightly in excess of what is expected by chance. Lenticule replicates performed by three other laboratories also produced satisfactory results. It is thought that lenticules could improve the accuracy of total plate counts and lead to a better standardization of quantitative methods in food microbiology within and between laboratories.

INTRODUCTION

There is an increasing demand for the provision of safety checks on food products and processes (Anon. 1988, 1995), especially in terms of their bacterial content. As well as screening for the presence or absence of defined pathogens, most routine surveillance programmes include an assessment of the total bacterial load (Gilbert 1992; Anon.1996). This is based on the premise that low counts imply better quality and lower risk and that high counts risk not only the possibility of food spoilage and reduced shelf-life, but also food poisoning organisms. There is a variety of available methods for the enumeration of organisms in foods, including pour plates (Anon. 1991a), most probable number (Anon. 1994), Miles and Misra (1938) and surface spread methods including spiral plating devices.

The spiral plating device was first described by Gilchrist *et al.* (1973) as a method that could be employed to enumerate pure cultures and inoculated milk and cream samples. The principal of spiral plating is that a constant volume of sample, or a dilution of it, is dispensed on a rotating agar plate in the form of an Archimedes spiral, the volume of the sample deposited decreasing across the spiral. As each area of the plate has a known deposition volume, the numbers of colony-forming units (cfu) produced in each sector are directly proportional to the original number of organisms per gram of food. Jarvis *et al.* (1977) evaluated the spiral plater in food microbiology and found that it produced comparable results with conventional methods with the added advantage of a 30% saving in operator time. Further time savings have been made by the introduction of mechanical and automated plate readers.

Laboratory accreditation bodies require that all methodologies and processes are regularly checked for accuracy and reproducibility (Anon. 1991b). This requirement applies especially to quantitative techniques, so it follows that reliable and reproducible controls are essential to facilitate this requirement.

The philosophy of having a countable number of organisms in food and water quality control is growing. Quantitative analysis allows for the detection of trends, consistency, reproducibility and deviations from acceptable parameters. A number of methods have been successfully employed in the production and storage of organisms, several of which utilize a drying process. Freeze drying has remained the principal method of preserving and maintaining organisms for over 50 years and has proved effective for a wide range of bacteria

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(Soriano 1970) and viruses (Rhoades 1970). Stamp (1947) described a method for the preservation of bacteria in the form of dried or freeze-dried gelatin discs. Some organisms proved difficult to store but with minor modifications to the medium, Obara *et al.* (1981) demonstrated that even fastidious organisms such as *Neisseria* and *Haemophilus* species can be stored using this method for quality control. Previous methods have described storage of organisms in terms of logarithmic survival (Rhoades 1970; Lapage and Redway 1974; Rudge 1984). This paper describes an alternative to current methods used in the preservation of biologically active materials which not merely secures survival, but also preserves quantitative activity for use as control material in food microbiology. The process devised for storage has been called lenticulation. Lenticules are control dried plano-convex discs containing biologically active material in a solid water-soluble matrix. The stability and activity of the materials, including viable organisms, is preserved over long periods of time.

MATERIALS AND METHODS

Lenticules

Lenticules for spiral plating were prepared from *Escherichia coli* NCTC 12950 in batches of 200. This strain was selected in the first instance in preference to NCTC 9001 and NCTC 10418 on the basis of its known long-term stability in lenticules. Subsequently, studies using NCTC 9001, the recommended PHLS control strain, have given satisfactory results. A pure culture of organism was produced by seeding on a supportive medium (CM3 Oxoid nutrient agar) and incubating aerobically at 37 ± 1 °C for 20 h. The yield from a single standard 90 mm Petri dish (about 10^{11} cfu) was emulsified in 250 µl saline solution, pH 7 ± 0·2, and mixed thoroughly with 2·5 ml lenticulating fluid (Codds Medium); 25 µl volumes of this suspension were spotted onto a hydrophobic surface (parafilm) and dried by forced ventilation in a desiccating cabinet for 18 h at 20 °C ± 2 °C followed by further drying at 4 °C ± 2 °C for 48–72 h over silica gel. During the drying period, the liquid spots change into the lens-shaped bodies or lenticules. These are the primary or storage lenticules, each containing approximately 10^8 or more live organisms. Primary lenticules were kept at −20 ± 2 °C over silica gel until either used to provide a fresh culture by plating out or to be rehydrated and mixed with more lenticulating fluid to make secondary lenticules with a defined countable number of organisms. Secondary lenticules were stored in a refrigerator at 2–8 °C. *Escherichia coli* lenticules for this study were produced to give a final count of $2·0 \times 10^5$ cfu lenticule^{-1}.

Culture media

Standard plate count agar, Oxoid CM 463, was used to enumerate *E. coli*. Plates were poured on a level surface and allowed to dry overnight at 2–8 °C prior to use. Sterile Saline-Peptone (Oxoid CM 733) in 225 ± 1 ml was used to resuspend prepared lenticules to form control material, and 9 ml Saline-Peptone were used to make serial dilutions from this.

Preparation of controls

Lenticules were removed from the refrigerator at 2–8 °C and left for 5 min before opening to attain room temperature and avoid condensation. The parafilm under the lenticule was flexed to free an edge; the lenticule was then removed with fine forceps and placed in 225 ml Saline-Peptone, the routine volume used to dilute standard 25 g samples for processing. This suspension was shaken for approximately 30 s and left to stand for 10 min. A further vigorous shake of the suspension for 30 s followed to allow dispersion of the organisms. This control material was treated exactly as a 1 in 10 dilution of stomached food, making further dilutions by transferring 1 ml sample serially to 9 ml Saline-Peptone. The dilutions were processed on the spiral platers in accordance with the manufacturer's instructions. Miles and Misra counts were performed in parallel with each of the control suspensions. All plates were left at room temperature for 15 min for the inocula to dry before inversion and incubation at 30 ± 1 °C for 48 ± 6 h.

Lenticules were also used in replicate trials. Five lenticules from the same batch were prepared as above and then 50 µl replicates used by both methods. The resultant cfu were counted by two independent observers. A further set of five lenticules was replicate tested by both methods for standardization following the same protocol. A series of three lenticules was posted to three external food laboratories each of which was asked to perform six replicate counts using a spiral plating process. Lenticules from the same batch were posted back to the sending laboratory and tested against stored lenticules.

Apparatus

Two models of spiral plating machine were used during the work: spiral plater model D (Don Whitley Scientific Ltd, Shipley, UK) was used up to 7 March 1997, and the Whitley automated spiral plater (WASP)(Don Whitley Scientific Ltd) from 3 March 1997.

Plates were counted manually with the aid of a Whitley colony counter throughout the study and from 23 April 1997, also with the Protos automated counter (Synoptics Ltd).

Calibrated Gilson pipettes (P1000) were used to make dilutions where required and a timer was used during the rehydration of the lenticule.

RESULTS

Table 1 shows the ranges and overall mean colony counts obtained by each method. Comparison of the spiral plater with Miles and Misra showed very similar mean counts in each batch. The overall correlation between the methods within a batch was very good and there was no evidence that the methods differed in the mean counts obtained across batches (mean difference = 0·04 $\log_{10}$ cfu with a 95% confidence interval of −0·10 to +0·01). The variation between batches was greater than within batches. This was as to be expected as batches were prepared on different dates and were run sequentially during the study. There was no change in the mean counts obtained throughout the in-use period of a given batch (13–30 weeks). Fourteen (2·5%) of the runs failed on the basis that the difference between the spiral plate and the Miles and Misra results was greater than 0·5 $\log_{10}$. This is the tolerance set out in the laboratory's standard operating procedure and approved by the accreditation body (UKAS). None of the failures had differences in counts greater than 1·0 $\log_{10}$ (Table 1). Ten of the 14 failures occurred in the first year of the trial.

In addition to the 596 complete sets included in the analysis, a further 17 runs could not be incorporated, mostly for technical reasons. On 10 occasions, the plate surface was too wet, rendering a surface count impossible. Twice the orifice of the spiral plater was blocked and once the machine malfunctioned. Inappropriate dilutions were used on three occasions and once, the control was omitted.

In order to examine variation within and between lenticules in more detail, five replicate counts (1–5) were obtained on each of five lenticules (A–E) from the same batch using the two counting methods. The results are shown in Tables 2 and 3. There appears to be good correlation between the methods (Pearson's correlation coefficient = 0·8) and they do not differ significantly in their mean counts (*t*-test *P*-value = 0·13). The second set of lenticules tested on a subsequent occasion gave almost identical results. If it is assumed that cfu were randomly spread in the dilution, and that there was no variation in the inoculum size and no variation in the method of counting, then it is possible to calculate that the standard deviation between counts on the same lenticule should be 6·3 (95% of counts for a lenticule within ±13 of the mean). The actual standard deviation within a lenticule is 8·4 for Miles and Misra and 7·5 for spiral plate (95% of counts for a lenticule are to be within approximately ±16 of the mean).

Variation between lenticules slightly exceeded variation

Table 2 Replicate counts (cfu) on five lenticules of *Escherichia coli* NCTC 12950 by Miles and Misra

	1	2	3	4	5	Mean
A	39	32	38	38	24	34·2
B	38	37	39	31	33	35·6
C	47	41	63	46	52	49·8
D	42	64	32	42	39	43·8
E	47	57	42	38	33	43·4

Overall mean = 41; S.D. within lenticules = 8·4, between lenticules = 9·9.

Table 3 Replicate counts (cfu) on five lenticules of *Escherichia coli* NCTC 12950 by spiral plater

	1	2	3	4	5	Mean
A	40	33	29	39	38	35·8
B	33	43	34	32	24	33·2
C	62	27	43	38	45	43·0
D	40	37	45	52	39	42·6
E	37	36	40	33	31	35·4

Overall mean = 38; S.D. within lenticules = 7·5, between lenticules = 8·0.

Table 1 Mean (and range) $\log_{10}$ colony count (cfu g^{-1}) of *Escherichia coli* NCTC 12950 in control sample

Batch no.		No. of runs by spiral plater	No. of runs by Miles and Misra	No. of operators
1	53	5·81 (3·90–6·74)	5·84 (3·66–6·36)	4
2	140	4·24 (2·30–5·30)	4·24 (2·00–5·34)	4
3	119	4·56 (2·90–5·04)	4·44 (2·90–5·07)	6
4	98	4·15 (2·61–4·90)	4·14 (2·60–4·87)	5
5	52	4·45 (2·85–4·91)	4·43 (2·91–4·88)	5
6	53	3·88 (3·00–4·66)	3·81 (2·85–4·58)	6
7	81	3·92 (2·60–4·61)	3·80 (2·84–4·60)	5

Total runs = 596; overall mean = 4·43 and 4·39 by the two methods.

within lenticules as would be expected. For Miles and Misra, the standard deviation is 9·9 and for spiral plate, 8·0. Therefore, 95% of counts on different lenticules in the same batch gave counts within approximately ±18 of the mean of 40. On the $\log_{10}$ scale, this is equivalent to counts being within −0·3 to +0·2 of the mean.

Table 4 demonstrates results of replicate testing by three independent food laboratories. Good correlation of results was achieved throughout.

DISCUSSION

The idea of preserving bacteria by a drying process is by no means new. Pasteur dried organisms on silk threads and garnets, and, the development of freeze drying has been the mainstay of keeping bacterial strain collections for many years. However, some organisms are notoriously difficult to preserve and in some instances, there may be a substantial reduction in numbers during the process (Rudge 1984). Impairment of growth characteristics and changes in genotypic and phenotypic markers have been described (Lapage and Redway 1974). Cryopreservation provides an alternative method, either as a liquid phase on beads at −20 °C, or frozen at −80 °C in a mechanical freezer. Storage in liquid nitrogen, especially for pathogenic species, is not recommended on the grounds of safety, although it is a very effective method (Lapage and Redway 1974). A limitation to low temperature storage is the inconvenience of maintaining a cold chain if these materials are to be transported without deterioration.

Storage of organisms by lenticulation circumvents many of these limitations. Lenticules can be prepared to contain a wide range of bacteria and in counts from 10^9 down to 10 cfu lenticule^{-1} (unpublished data). Reconstitution from the lenticule to the culturable form requires only 10 min hydration either by direct contact on a solid medium or in a solution. As it is possible to produce lenticules with a closely defined count of live organisms, it is convenient to make them for single use without the need for division or dilution of the suspension. This differs from the standard operating procedure in the production of control material and is arguably simpler and less prone to error. Variation within and between lenticules is low and only slightly exceeds what is expected by chance. Results from the replicate testing confirm the reliability of the counting methods used and also demonstrate the reliability of the lenticules in providing reproducible inocula. Naturally, accuracy is obtained by selecting a dilution that contains the optimal countable number. Too small a number and the range becomes too wide. Too heavy an inoculum and the count cannot be made with ease or precision. The effect of observer error was also investigated by using two operatives to count the replicates. There were very few discrepancies and in fact the totals across the five replicates did not differ. It would appear that lenticules remain stable following normal postal conditions, giving consistent and reproducible counts on replicate testing. It is recognized that further testing will be required to validate this. The colonial morphology, cultural and biochemical characteristics appear unimpaired by lenticulation. It is essential that lenticules are kept completely dry during storage and transportation. This can be accomplished by the inclusion of silica gel within a well-sealed transport carrier. Lenticules are best stored at −20 °C but, in this study, they were stored between 2 and 8 °C without any significant reduction in viability. If care is taken to prevent rehydration and excessive temperature fluctuations, shelf-life has been shown to be in excess of 3 months. Storage shelf-life at −20 °C is greater than 2 years for the organism used in the study.

A number of operators used the lenticule system during this study and results obtained were consistent irrespective of user. All operators commented on ease of use. However, there were two criticisms. Firstly, some of the initial lenticules produced were too soft, making handling difficult. This was not a problem with later batches. Secondly, clear lenticules were sometimes difficult to see. To this end, coloured lenticules using non-inhibitory dyes are being developed. Colour provides the option of an additional identity check.

Crucial to the assurance in accuracy of the spiral plating technique is the provision of suitable controls. As well as daily electro-mechanical checks, performance controls using dyes and bacterial suspensions are recommended. The dispensed volume is of course critical to the final calculation and ultimately the test result. If volume constants or the sensitivity and reproducibility of a given machine are to be determined, then several suspensions and/or dilutions may be required. For accurate results to be obtained, a minimum of 20 colonies must be present on each plate (Gilchrist 1977). It therefore follows that bacterial controls must be reliably above this threshold and yet stay within the countable range of the process. Lenticules clearly fulfil this requirement.

Table 4 Spiral plate counts from three independent food laboratories. Mean $\log_{10}$ counts from six replicate lenticules of *Escherichia coli* NCTC 12950

	Lenticule		
Laboratory	A	B	C
1	4·41	4·45	4·53
2	4·05	4·09	4·09
3	4·08	4·07	4·11
Sending laboratory	4·29	4·52	4·41

Spiral plating is not the only process that would benefit from a control challenge with a defined number of organisms. The recovery of low levels of salmonellas, listeria and *E. coli* O157 could be validated and uncertainty measurements calculated using lenticules. It must be recognized, however, that organisms in true samples may be sub-lethally damaged and that measurements of uncertainty using lenticules will be those under ideal conditions.

Lenticules provide a dependable and convenient alternative to wet cultures for quality assurance and process controls in routine food microbiology. It is likely that they may prove beneficial to microbiologists in other areas of quality assessment and assurance.

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